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Evaluation of a new method of selective dry cow treatment using microbiological culture and antibiogram results

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Abstract

Background Due to financial issues and the rise in antimicrobial resistance, updating dry cow therapy (DCT) methods is still being researched by scientists worldwide. This investigation aimed to evaluate a new method of selective treatment by choosing an appropriate dry cow antimicrobial product for each cow based on the individual culture and antibiogram results and examining its effects on clinical and subclinical mastitis indices, cure rate, new infection rate, and milk yield during the first 30 days in milk (DIM).

Materials and methods A total of 291 Holstein dairy cows were selected from three herds. These cows had a somatic cell count (SCC) > 200,000 (cells/mL) just before drying off, had positive composite milk culture results, and were gradually dried over five days. The milk samples were taken before drying off and on the day after calving (1 DIM) for microbial culture evaluation, as well as 3 days before drying off and between 3 and 7 days postpartum to evaluate SCC. The cows were randomly divided into two groups of control ($n=151$) and treatment ($n=140$). The control group included cows that were treated with dry cow antimicrobial products regardless of the pathogens involved in mammary infection, and the treatment group contained cows that received dry cow antimicrobial products based on the type of pathogen isolated during culture and the antibiogram results before drying off.

Results The results revealed that the cure rate in the treatment group was significantly better than that in the control group ($P=0.0006$). In addition, the rate of new intramammary infections (IMI, $P=0.0006$) and the rate of clinical mastitis ($P=0.015$) decreased in the first 30 DIM in the treatment group. Nevertheless, the SCC and milk yield at the onset of subsequent lactation did not differ significantly between the control and treatment groups ($P>0.05$).

Conclusion According to the findings of our study, based on individual milk culture and antibiogram results, selectively treating cows with appropriate dry cow antimicrobial products had significant benefits for increasing the cure rate of pathogens, lowering the incidence of new IMIs, and minimizing the risk of clinical mastitis in the first 30 DIM.

Keywords Mastitis, DCT, Pathogen, Antibiogram, SCC

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Background

Mastitis is an inflammation of mammary gland tissue, and bacteria are the most common cause of this inflammation [1, 2]. In addition to treatment expenses, mastitis can result in substantial economic losses due to decreased milk yield and penalties associated with decreased milk quality [3]. Approximately 60% of all antimicrobials used on dairy cow farms are for the prevention and treatment of intramammary infections (IMIs), with two-thirds of that quantity relating to the DCT [4]. In the 1960s, DCT was proposed as a management strategy to reduce the prevalence of IMIs in subsequent early lactation [5], as the occurrence of IMIs during the dry period poses an increased risk of clinical mastitis after calving in subsequent lactation [6]. The National Mastitis Council recommends the infusion of a long-acting intramammary antimicrobial into all quarters of all eligible dry cows as a mastitis control strategy, particularly for contagious mastitis agents at drying off, called blanket dry cow therapy (BDCT) [7]. It is essential to manage dry cows from both the standpoints of eliminating existing infections (bacteria from previous lactation) and preventing new infections during dry period [8, 9]. The general treatment of cows has been crucial for lowering the prevalence of contagious mastitis and, consequently, the number of bulk tank somatic cells [9–11]. The probability of new infections in dairy cows depends on bacterial exposure, mammary defense mechanisms, and antimicrobial treatment at drying off [12]. It has also been determined that many cases of mastitis in early lactation are caused by dry-period infections; therefore, antimicrobial compounds continue to play an essential role in controlling mastitis during the dry period, and several antimicrobial compounds have been developed specifically for this purpose [8, 13, 14]. Dry cow antimicrobial products are administered at drying off, after the cow's last milking, and are highly effective against contagious pathogens; consequently, the number of quarters of milk samples with negative culture results at drying off has increased from 42.2% in 1985 to 73–95% in recent years [15, 16]. However, new IMIs can still occur despite DCT if the pathogens are resistant to the antimicrobials used or at the end of the dry period when there is a decrease in the concentration of intramammary antimicrobials [17, 18]. On the other hand, the increasing trend of indiscriminate use of antimicrobials on dairy farms has increased the likelihood of antimicrobial resistance [2, 19, 20]. As a result, antimicrobial compounds, as triggers for the development of antimicrobial resistance, have prompted additional research to limit their indiscriminate use [17, 21]. With the growing emphasis on the prudent use of antimicrobial compounds and the concern about the emergence of antimicrobial resistance, the treatment procedure for each cow at the end of lactation has been called into question. The prudent use of antimicrobial compounds in medicine, veterinary medicine, and agriculture is emphasized, and the European Commission advises avoiding routine antimicrobial therapy of cows at drying off [22]. As a result, some countries have implemented legal restrictions on the excessive use of antimicrobials [2, 5]. The issue of updating the DCT recommendations in response to the worldwide problem of antimicrobial resistance is ongoing. An alternative to BDCT for controlling mastitis at drying off is targeted drying off only in cows with IMI, which is termed selective DCT (SDCT). The most effective method for selecting cows and quarters for selective treatment is milk culture [23, 24]. Various studies have also utilized individual SCC tests of cows, California mastitis tests, and milk leukocyte differential tests [25–27]. Due to the complexity of mastitis and the variety of microorganisms that cause this inflammation, an emphasis is placed on the specific treatment of each factor [28]. The method used to select cows for drying off with antimicrobial products should be precise, practical, relatively secure, and interpretable. Only two methods, milk microbial culture and SCC evaluation before drying off, appear to be close to meeting these criteria [29]. Many laboratories throughout the globe continue to isolate bacteria and diagnose mastitis agents using milk culture [30]. In addition, on-farm culture systems are commonly utilized to make strategic treatment decisions and facilitate the management of mastitis based on the type of pathogen isolated from dairy cow herds [31, 32], which can reduce the use of antimicrobial without compromising cow health [32]. During the lactation period, antimicrobials are used to treat clinical and subclinical mastitis on dairy farms. However, at drying off, antimicrobial infusion is administered in all quarters without an accurate diagnosis of the cause of mastitis [21]. Selective therapy significantly reduced the use of antimicrobials without adversely affecting SCC [33, 34], clearing IMIs, preventing new infections during the period, culling rate [23, 35], the risk of clinical mastitis during the subsequent lactation period, and milk yield [36, 37].

According to reports, in 10–40% of clinical mastitis cases, no pathogenic agent develops in bacterial cultures; therefore, these cases do not require antimicrobial treatment. Furthermore, 40% of positive culture cases (gram-negative organisms and yeasts) are resistant to antimicrobials in intramammary products [38]. The purpose of SDCT is to administer dry cow antimicrobial products based on cow mastitis history including at least the knowledge about cow somatic cell count, but also, when possible, the pathogens causing mastitis during the lactation. Therefore, our hypothesis for this study was that the efficacy of the DCT would increase if dry cow antimicrobial products were administered based on the

Table 1 The microbiological isolation information before drying off and post-calving sampling

Item	Groups	1 pathogen	2 pathogens	No growth	Total pathogen count
Before drying off	Control	146	5	0	156
	Treatment	133	7	0	147
Post-calving	Control	103	20	28	143
	Treatment	58	5	77	68

culture and antibiogram results, and it would have a positive effect on reducing clinical and subclinical mastitis in the first 30 days of subsequent lactation.

Results

The prevalence of pathogens in the study

Before drying off, a total of 392 cows were sampled, and pathogens were isolated from 74% (291/392) of the cows. The total prevalence rates of *Non-aureus staphylococci (NAS)*, *Staphylococcus aureus* (*Staph. aureus*), *Corynebacterium bovis* (*C. bovis*), *Escherichia coli* (*E. coli*), *Pseudomonas*, *Streptococcus dysgalactiae* (*Strep. dysgalactiae*), *Enterobacter*, *Serratia*, *Proteus*, *Pasteurella*, *Klebsiella* and *Streptococcus uberis* (*Strep. uberis*) before drying off were 26.80% (78/291), 24.74% (72/291), 19.24% (56/291), 15.46% (45/291), 7.21% (21/291), 2.74% (8/291), 2.40% (7/291), 2.06% (6/291), 1.37% (4/291), 1.37% (4/291), 0.34% (1/291) and 0.34% (1/291), respectively. Additional information can be found in Table 1.

Among the milk cultures collected from post-calving cows, 36.08% (105/291) had no isolated pathogens. The total prevalence rates of *NAS*, *Staph. aureus*, *C. bovis*, *E. coli*, *Pseudomonas*, *Strep. dysgalactiae*, *Pasteurella*, *Klebsiella*, and *Strep. uberis* were 24.05% (70/291), 23.71% (69/291), 10.65% (31/291), 9.96% (29/291), 1.03% (3/291), 1.71% (5/291), 0.34% (1/291), 0.34% (1/291), and 0.68% (2/291), respectively (Table 1). In our study, we isolated two pathogens from several samples. The microbiological isolation information is presented in Table 2.

If one or two pathogens were isolated from the milk sample, it was included in the statistical analysis, but if ≥ 3 pathogens were isolated, the sample was considered contaminated and excluded from the study.

Table 2 The number of pathogens isolated before drying off and post-calving sampling, new infections, and the cure rate

		Pathogen count (*n*)		Prevalence (pathogen/cow) (%)			
Item	Group	Before drying off	Post-calving	Before drying off	Post-calving	New infection (pathogen/cow) (%)	Cure rate (presence before drying off but absence post-calving) (%)
NAS	Control	34	43	22.51	28.47	15.89	38.23
	Treatment	44	27	31.42	19.28	8.57	65.90 *
Staph. aureus	Control	31	50	20.52	33.11	19.20	32.25
	Treatment	41	19	29.28	13.57	5.71	73.17 *
C. bovis	Control	30	20	19.86	13.24	4.63	56.66
	Treatment	26	11	18.57	7.85	4.28	80.76 *
E. coli	Control	26	19	17.21	12.58	5.96	61.53
	Treatment	19	10	13.57	7.14	4.28	78.94 *
Pseudomonas	Control	16	2	10.59	1.32	0.00	87.50
	Treatment	5	1	3.57	0.71	0.71	100.0
Strep. dysgalactiae	Control	3	5	1.98	3.31	3.31	100.0
	Treatment	5	0	3.57	0.00	0.00	100.0
Enterobacter	Control	5	0	3.31	0.00	0.00	100.0
	Treatment	2	0	1.42	0.00	0.00	100.0
Serratia	Control	5	0	3.31	0.00	0.00	100.0
	Treatment	1	0	0.71	0.00	0.00	100.0
Proteus	Control	2	0	1.32	0.00	0.00	100.0
	Treatment	2	0	1.42	0.00	0.00	100.0
Pasteurella	Control	3	1	1.98	0.66	0.00	66.66
	Treatment	1	0	0.71	0.00	0.00	100.0
Klebsiella	Control	1	1	0.66	0.66	0.00	0.00
	Treatment	0	0	0.00	0.00	0.00	0.00
Strep. uberis	Control	0	2	0.00	1.32	1.32	0.00
	Treatment	1	0	0.71	0.00	0.00	100.0

* Indicates a significant difference between the control and treatment groups ($P < 0.05$)

Cure rate

The overall cure rate was 75% (105/140) in the treatment group and 54.96% (83/151) in the control group, which was significantly greater in the treatment group ($P = 0.0006$). The cure rates for specific pathogens in the treatment group were significantly greater than those in the control group ($P < 0.0001$). In the treatment group, the cure rates for *NAS*, *Staph. aureus*, *C. bovis*, and *E. coli* were 65.90%, 73.17%, 80.76%, and 78.94%, respectively. In the control group, the cure rates for *NAS*, *Staph. aureus*, *C. bovis*, and *E. coli* were 38.23%, 32.25%, 56.66%, and 61.53%, respectively (Table 1).

The new infection rate

The overall new infection rate in the treatment group was 22.8%, which was significantly lower than that in the control group (42.4%, $P = 0.0006$, Table 1).

The incidence of clinical mastitis

The incidence of clinical mastitis during the first 30 DIM in the control group (17.2%) was significantly greater than that in the treatment group (7.8%, $P = 0.015$). Notably, the levels of SCC and milk production at the time of drying off and at the beginning of lactation did not differ between cows in to the control and treatment groups ($P > 0.05$). Additional information is shown in Table 3.

Discussion

BDCT at the end of lactation is being scrutinized due to the increasing focus on judiciously using antimicrobial compounds and the rising concern about developing antimicrobial resistance [39]. The effectiveness of an SDCT approach can be evaluated by the degree of reduction in the use of antimicrobials at drying off, the absence of any negative impact on the incidence and elimination of IMIs at drying off, and improvements in udder health and milk production during subsequent lactation [34]. The success of this approach depends on the ability to accurately determine the infection status of the cow, which will help with the appropriate drying off treatment. Apart from selecting only the cows that require DCT, it is important to choose the most appropriate antimicrobial for each cow, and this will be a crucial step in the targeted treatment of cows. Hence, in this study, cows with an SCC > 200,000 cells/mL and a positive composite milk culture result were selected, and in the control group, the routine SDCT program was used; however, the dry cow antimicrobial products in the treatment group were chosen according to the antibiogram results.

A new method of SDCT effects on the cure rate

Our study demonstrated that DCT based on antibiogram results can enhance the effectiveness of SDCT and

Table 3 Evaluation of factors related to herd health before drying off and post-calving on the studied farms

		SCC (× 1000) (cells/mL)		Milk yield (kg)		SCC > 200/000 (cells/mL) (%, *n*)		Clinical mastitis (%, *n*)	Parity	Number
		Before drying off	Post-calving	Before drying off	Post-calving	Before drying off	Post-calving	Post-calving		
Farm 1	Control	318.3	229.7	28.5	39.5	100 (50/50)	42 (21/50)	16 (8/50)	1 2 3 ≥4	0 27 13 10
	Treatment	309	239	30.7	37.7	100 (51/51)	27 (14/51)	5 (3/51)	1 2 3 ≥4	0 19 12 20
Farm 2	Control	320.9	302.3	24.2	35.8	100 (50/50)	36 (18/50)	16 (8/50)	1 2 3 ≥4	2 13 17 18
	Treatment	540.9	284.8	22.3	32.6	100 (37/37)	35 (13/37)	8 (3/37)	1 2 3 ≥4	3 5 11 18
Farm 3	Control	449.5	274.3	31.6	42.5	100 (51/51)	35 (18/51)	19 (10/51)	1 2 3 ≥4	0 19 11 21
	Treatment	311.4	157.4	28.7	43.6	100 (52/52)	19 (10/52)	9 (5/52)	1 2 3 >4	1 14 18 19

lead to improved cure rates. The successful outcome of an SDCT program depends on the accurate diagnosis of infection status and the type of pathogen at drying off so that DCT can be applied appropriately and judiciously to eliminate the existing IMI [24]. Despite reducing the use of antimicrobials and increasing the cure rate, the SDCT method has not negatively affected the treatment of IMIs and udder health [24, 34, 35]. Cameron et al. [17] reported that the cure rates for BDCT and SDCT were similar regardless of the Petrifilm culture results, which were 84.5% and 89.0%, respectively. In the studies of Rowe et al. [18], the cure rates of IMI in the BDCT, culture-guided SDCT, and algorithm-guided SDCT groups were 87.33%, 88.71%, and 88.12%, respectively. Patel et al. [23], reported that the cure rates for culture-guided SDCT and BDCT were 82.3% and 88.0%, respectively. The proportion of quarters experiencing a cure between dry off and 0 to 10 DIM was 84.8% and 85.7% for dry cow antimicrobial products including cloxacillin benzathine and ceftiofur hydrochloride, respectively [40]. In our study, the cure rate in both the control and treatment groups was lower than that in the studies we referenced. One of the main reasons for the lower cure rate is the difference in the criteria for selecting cows to participate in the study. In our research, cows were included if they had an SCC > 200,000 cells/mL and a positive culture result, indicating more chronic infections that were less likely to be cured. In the studies we referenced, only one of these criteria was required for selecting cows, which may be more effective in increasing the overall cure rate. Notably, the cure rate can be affected by the type and proportion of pathogens causing IMIs. Nearly a quarter of the pathogens identified in our research were associated with multidrug-resistant bacteria, such as *Staph. aureus*; however, in the studies conducted by Cameron [17], Rowe [18], Patel [23], and Johnson [40], the prevalence of *Staph. aureus* was 2.64%, 1.02%, 6.89%, and 1.15%, respectively.

The results of our study revealed that this treatment approach had no significant effect on average milk yield or SCC. Cameron et al. [17, 24] reported that the average milk yield and SCC during 180 DIM did not differ substantially between the BDCT and SDCT groups based on the results of Petrifilm culture. Similarly, Vasquez et al. [35] found no significant difference between the average daily milk yield and the linear score of SCC in the BDCT and SDCT groups. Consistent with these studies, Kabera et al. [34] observed no statistically significant difference in the average daily milk yield or the linear score of SCC in the first 120 DIM based on Petrifilm culture results. Various factors can influence the outcomes of this study. The Bacteriological cure rate is expected to rise as parity decreases [41]. Moreover, an increase in milk production and metabolic stress may lead to a decrease in the cure rate [42]. The location of infection in the mammary gland varies among different bacterial strains, and their virulence characteristics and immunogenic capabilities can also differ. Therefore, the type of virulence factor of pathogens also plays a significant role in the cure rate [43].

The cure rate of individual pathogens

Treatment based on the antibiogram results could significantly increase the cure rate of *NAS* (65.90%), *Staph. aureus* (73.17%), *E. coli* (78.94%), and *C. bovis* (80.76%). However, there was no significant difference in the cure rate for the other pathogens between the control and treatment groups due to an insufficient number of pathogens. Overall, the cure rate improved because of an increase in the cure rate of the mentioned pathogens. In the study by Pantoja et al. [16], the cure rates after DCT for *NAS*, *Streptococci* and *Coryneforms* were 61.5%, 23.1%, and 9.9%, respectively. According to the study conducted by Schmenger et al. [44], bacteriological cure rates for *Staph. aureus*, *Coliforms*, *NAS*, and *Strep. dysgalactiae* were 44.7%, 87.1%, 77%, and 82.9%, respectively. In the study by Johnson et al. [40], the cure rate after DCT for *NAS*, *C. bovis*, *Staph. aureus*, and *E. coli* were 60.38%, 100%, 75%, and 53.33%, respectively.

Staph. aureus is a multidrug-resistant bacteria, and it's low cure rate, making it one of the main causes of post-calving IMI [45]. According to Shephard et al. [46], the cure rate for DCT of *Staph. aureus* was 47.7%. However, in a more recent study conducted in Iran, Amiri et al. [47] reported a greater *Staph. aureus* cure rate of 58.1% after DCT. The antimicrobial resistance patterns of *Staph. aureus* strains isolated from bovine subclinical mastitis were evaluated in Alborz Province, Iran, and 38 (84.4%) *Staph. aureus* isolates were resistant to at least 3 antimicrobial agents. Therefore, performing antimicrobial susceptibility tests before antimicrobial compound prescription seems necessary [48].

The new infection rate

In our study, all the included cows had an SCC > 200,000 cells/mL and a positive composite milk culture, and if the bacterium isolates in the composite milk sample at drying off differed from those found post-calving, it was considered a new infection. As a result of the need to improve DCT methods, our findings showed that the rate of new IMIs post-calving was significantly lower in the treatment group. According to the study by Vanhoudt et al. [49], the new infection rates according to the SDCT method ranged from 16 to 18%. In contrast, Vasquez et al. [35] reported that the new infection rate was lower among low-risk cows, with an SCC less than 200,000 cells/mL, at only 5.5%. The occurrence of new IMIs may be influenced by parity, genetics, SCC, infection with minor mastitis pathogens, and teat characteristics [50].

It is important to note that the development of antimicrobial resistance after antimicrobial treatment can occur not only in pathogenic bacteria but also in innocent bystander microorganisms that make up a body system, such as the mammary gland [51]. Based on the significant reduction in new infections in the treatment group, the correct selection of antimicrobials likely has less impact on the intramammary microbiota and thus can significantly lower the rate of new IMIs post-calving. It's important to note that after DCT, a cow could be cured of an infection during the dry period, only to become reinfected with the same pathogen during the dry period or shortly after calving. Therefore, it is recommended to use genotyping techniques to identify similar strains in future studies in this field.

The incidence of clinical mastitis

The incidence rate of clinical mastitis ranges from 13 to 40% per year in different countries and housing types [52–54]. Clinical mastitis during the first 30 DIM can be caused by untreated IMIs at drying off or new infections after DCT [55]. According to Green et al. [56], 38% of clinical mastitis cases during lactation were associated with microorganisms that were isolated from milk samples at drying off. This highlights the importance of effective dry cow management and treatment to prevent and control mastitis during subsequent lactation. Van den Borne et al. [54] reported that during the first 30 DIM, the incidence of clinical mastitis was estimated to be 4.6 and 2.0 times greater than that during the remaining lactation in multiparous and primiparous cows, respectively. In another study, clinical mastitis incidence rates were 20.2 cases per 100 cow-months at risk in the first 30 days of lactation and 4.3 cases per 100 cow-months at risk for the remainder of the lactation period [57]. A recent study by Rowe et al. [18] showed a 13.7% risk for clinical mastitis in the SDCT group during the first 120 DIM. Based on our results, the incidence of clinical mastitis within 30 DIM was significantly greater in the control group than in the treatment group, which is noteworthy because our inclusion criteria for cows were SCC > 200,000 cells/mL and a positive composite milk culture. Therefore, selecting the dry cow antimicrobial products at drying off based on the antibiogram results will significantly decrease the incidence of clinical mastitis post-calving, which, in addition to reducing the use of antimicrobials, can decrease economic losses caused by the decline in milk production and the costs of treatment or culling.

Conclusion

The application of a program that uses antibiogram-based SDCT is beneficial for public health because it minimizes the potential for antimicrobial resistance. Using the antibiogram-based SDCT approach to make targeted treatment decisions on cows with SCCs greater than 200,000 cells/mL at the end of lactation resulted in increased cure rates after dry period and a decreased prevalence of new intramammary infection, as well as a reduced risk of clinical mastitis in the first 30 DIM compared to SDCT. Further analysis of the trial data will cover the economic aspects of antibiogram-based SDCT.

Methods

Herd selection

This randomized clinical trial was conducted on three large dairy cow herds with 600, 700, and 1,500 milking dairy cows under the administration of one unit in Mashhad Province, Iran. In all herds, the cessation of lactation was performed gradually over 5 days alongside the change in diet, and DCT was performed in the form of BDCT. The cows were housed in free-stall facilities, were milked three times per day, and had respective daily milk records of 39, 40, and 43 kg. Under the supervision of nutritionists, the cows received a total mix ratio diet containing corn silage, alfalfa, concentrate, and supplements three times daily. Table 4 contains additional information about the herds and cows.

Selection of cows and random allocation to control and treatment groups

To the best of our knowledge, this study was the first to examine the treatment of IMIs using culture and antibiogram results. It's important to note that this was a preliminary study and we used a convenience sample, aiming for approximately 50 cows in both the control and treatment groups on each farm. This research was conducted

Table 4 Descriptive statistics for herds and cows enrolled in the study

Item	Farm 1	Farm 2	Farm 3	Total
Province	Mashhad	Mashhad	Mashhad	
Herd size (milking cows)	600	700	1500	2800
Housing type	Free stalls	Free stalls	Free stalls	
Control groups (cows)	50	50	51	151
Treatment groups (cows)	51	37	52	140
Average daily milk yield (kg)	39	40	43	40.6
Post-milking teat dipping	Iodine compounds	Iodine compounds	Iodine compounds	
Milking frequency (daily)	3 times	3 times	3 times	

Table 5 Descriptive information regarding the dry cow antimicrobial products and internal teat sealant used in the study

Dry cow antimicrobial products and internal teat sealant	Pharmaceutical composition
Tridry® DC (Kimia Biotechnology Co.)	Each 10 g: containing Cloxacillin (benzathine) 500 mg, Sulfadimidine (sodium) 750 mg, Trimethoprim 125 mg, Oily base q.s. 10 g
Spectramast® DC (Zoetis Co.),	Each 10 ml: containing Ceftiofur Equivalents (as the hydrochloride salt) 500 mg, Microcrystalline Wax 700 mg, Oleoyl Polyoxylglyceride 500 mg, Cottonseed Oil q.s.
Nafpencin® DC (Kimia Biotechnology Co.)	Each 10 gr: containing Penicillin G procain 300/000 I.U., Dihydrostreptomycin (sulfate) 100 mg, Nafcillin (sodium) 100 mg, Oily base q.s. 10 g
Cephapirin® DC (Afarin Darou Co.)	Each 8 gr: containing 300 milligrams of Cephapirin (benzathine)
Cloxamp® DC (Tolide Darouhaye Dami Iran Co.)	Each 5 gr: containing 500 mg of cloxacillin benzathine and 250 mg of ampicillin trihydrate
Masti seal® DC (Afarin Darou Co.)	Each 4 gr: containing bismuth subnitrate

on 291 Holstein dairy cows. The inclusion criteria at the cow level consisted of SCC > 200,000 cells/mL, a positive composite milk culture result 2–3 days before drying off, no evidence of clinical mastitis 2–3 days before drying off, no antimicrobial treatment within the last 14 days, ≥ 3 functional quarters, and a dry period of no less than 30 days and no more than 90 days.

The selected cows were randomly assigned to one of two control or treatment groups. In all the control groups, a conventional dry cow antimicrobial product (Tridry® DC, Kimia Biotechnology Co.) was administered, whereas the choice of dry cow antimicrobial product was based on the antibiogram results of the treatment groups. Based on the sensitivity or resistance of each pathogen to different antimicrobials, we selected appropriate dry cow antimicrobial products. If we isolated two pathogens simultaneously from the milk sample, we chose dry cow antimicrobial products based on the antibiogram results of the major pathogen. In both the control and treatment groups, we used Masti seal® DC (Afarin Darou Co.) along with dry cow antimicrobial products. More information is provided in Table 5.

Sampling at drying off and post-calving

The cow composite milk samples were collected as per the National Mastitis Council protocol [58]. Teats were disinfected, and the cow composite milk samples were collected in sterile tubes 2–3 days before drying off and on the day after calving for culture evaluation. The cow composite milk samples were collected 2–3 days before drying off and at intervals of 3–7 DIM for SCC evaluation. The samples were transported with ice to the laboratory and stored in the refrigerator for less than 48 h until culture.

Bacterial culture of samples

The milk culture procedures followed published procedures recognized by the NMC for bovine mastitis [59]. Before culturing, the sample was kept at room temperature until it was at the same temperature, and then approximately 0.01 mL of the sample was deposited in blood agar and McConkey agar media. Then, the samples were incubated at 37 °C and evaluated 24 h later, after which they were subjected to Gram staining. The catalase test was used to differentiate *Streptococcus*, *Staphylococcus*, and *Corynebacterium*. The appearance of white to gray and dry colonies and the absence of hemolysis in the blood agar were utilized if gram-positive *Bacillus* and *Coccobacillus* were detected. Gram-negative bacteria were identified using triple sugar agar, Simmons' citrate agar, and motility test media [59].

Microbiological outcomes

If two pathogens were isolated simultaneously from the milk sample, both results were considered acceptable for statistical analysis. In such cases, the selection of antimicrobials for the treatment group was based on the major pathogen. Samples with 3 or more differing isolates were classified as contaminated, and the associated cows were removed from the analysis [60]. For *Staph. aureus*, obtaining a negative culture result from a single sampling does not guarantee a successful cure. It is recommended to take two samples with an interval of one or two weeks to ensure a more accurate and reliable culture result. However, taking samples with the same interval also carries the risk of a new infection occurring after calving. A previous study that evaluated the relative sensitivity and specificity of diagnosing IMI using a single sample (vs. multiple samples) reported that triplicate samples collected over consecutive days provided only a modest gain in specificity and little or no gain in sensitivity as compared with a single sample [61]. In our study, if *Staph. aureus* was isolated from the cows' composite milk samples at drying off sampling, then two samples were taken from those cows on the day after calving sampling. One of the samples was directly cultured, and the second sample was frozen. If the result of the direct culture was negative, the second sample was cultured after 48 h, and if the result of the second culture was negative, it was considered cured.

Antimicrobial susceptibility test (AST)

AST was performed according to the disk agar diffusion method and Clinical and Laboratory Standards Institute

guidelines. For this purpose, a bacterial suspension with a turbidity of 0.5 McFarland was prepared and subsequently inoculated onto Mueller-Hinton agar media in three different orientations. The samples were then incubated at 37 °C. The inhibitory zone of each antimicrobial was measured using a ruler following a 24-hour incubation period. The antimicrobial disks of penicillin (10 units), ampicillin (10 µg), sulfadimidine trimethoprim (1.25/23.75 µg), streptomycin (10 µg), cefazolin (30 µg), ceftriaxone (30 µg), and cloxacillin (1 µg) were utilized in this study.

Somatic cell count

The direct microscopic somatic cell count was used for counting somatic cells. This method involved using a slide stained with a modified Newman-Lampert formulation of Methylene Blue milk smear stain. Four rectangles, each with dimensions of two centimeter by half a centimeter, were carefully drawn onto a microscopic glass slide. The milk samples were then mixed gently for twenty seconds before being evenly distributed onto each rectangle in amounts of ten microliters. The slides were then allowed to dry before being stained with the modified Newman-Lampert formulation of Methylene Blue milk smear stain. The cells were counted under a microscope with a 40X objective lens. To calculate the somatic cells in 1 ml of milk, the equation $N = n \times W$ was used, where n is the number of counted somatic cells and W is the work coefficient. The work coefficient (W) was calculated using the equation $W = (20 \times 100) / (d \times b)$, where d is the diameter of the microscope's field of vision (in mm) and b is the number of counting stripes (the lines from top to bottom of the visual field) [62].

Definitions

Cure rate The cow was deemed cured if the isolated pathogen in the cow composite milk sample before drying off were absent in the cow composite milk sample on the day after calving. The cow was deemed uncured if the pathogen isolated before drying off was detected in the composite milk sample on the day after calving.

New infection rate If the isolated pathogen before drying off the composite milk sample differed from those isolated on the day after calving, it was deemed a new infection.

Registration of clinical mastitis cases Any visible changes in milk with or without mammary inflammation, swelling, or redness in a quarter with or without systemic symptoms in the first 30 days of subsequent lactation were recorded as clinical mastitis cases. Additionally, each case of clinical mastitis was defined based on the affected quarter. If the same quarter was affected again after 14 days, a new case of clinical mastitis was considered.

Statistical analysis

The cows' parity and milk yield data were collected using the farm management system. Continuous data, including SCC and milk production at the time of drying off and at the beginning of lactation, were analyzed using the GLM procedure. Binary variables, including the rate of clinical mastitis, rate of new infections and cure rate at the beginning of lactation, were analyzed using the GENMOD procedure (logistic regression) with the logit link. It is worth mentioning that all analyses were initially performed without considering the species of the pathogens, and then, the analyses were conducted for each individual pathogen. All analyses were conducted in SAS version 9.4 (SAS Institute Inc., Carry, NC, USA). Differences at $P < 0.05$ were considered significant.

Abbreviations

DCT	Dry cow therapy
DIM	Days in milk
SCC	Somatic cell count
IMIs	Intramammary infections
BDCT	Blanket dry cow therapy
SDCT	Selective dry cow therapy
NAS	Non-aureus staphylococci
Staph. aureus	Staphylococcus aureus
C. bovis	Corynebacterium bovis
E. coli	Escherichia coli
Strep. dysgalactiae	Streptococcus dysgalactiae
AST	Antimicrobial susceptibility test

Supplementary Information

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Supplementary Material 1

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Author contributions

Research conceptualization, resources, supervision, writing-review & editing: MV, BK, FG, MTG; Samples collection and experiment performance: BK, AM; Formal analysis; VA; Original manuscript preparation: HN, AM; All the authors reviewed and approved the final manuscript.

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Data availability

All data supporting the findings of this study are available within the paper and its Supplementary Information.

Declarations

Ethics approval and consent to participate

This study was conducted on cows and informed consent was obtained from all owners. Protocol of study was confirmed by animal welfare committee of University of Tehran Veterinary Ethical Review Committee (IR.UT.VETMED.REC.1402.064) in accordance with institutional and national or international guidelines.

Consent for publication
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